Computer aided identification of toxicologically relevant substances by means of multiple analytical methods

Hartstra, Jan

Chapter 7

Inter-laboratory evaluation of an RP-HPLC method and refinement of the accompanying retention calibration procedure

7.1 Introduction

In this chapter, an HPLC system and its calibration method were evaluated for use in inter-laboratory databases suitable for substance identification in STA. The gradient RP-HPLC system for the screening and/or identification of toxicologically relevant substances developed by Bogusz *et al.* [67, 68], was selected. This system uses the 1-nitroalkanes as primary calibration standards (*i.e.* as a retention index scale) [71], and selected reference drug mixtures as secondary calibration standards [142, 143].

The evaluation and refinements are based on inter-laboratory experiments using different batches of a single type and brand of column, and by utilizing a fixed set of test substances that was the same in all laboratories.

7.2 Experimental

7.2.1 Setup

The experiments were performed in three different laboratories:

Groningen: Department of Analytical Chemistry and Toxicology, University Centre for Pharmacy, University of Groningen, A. Deusinglaan 1, NL-9713 AV Groningen, the Netherlands (Prof dr. R.A. de Zeeuw, Dr. J.P. Franke, J. Bosman, J. Hartstra).

Cracow: Institute of Forensic Medicine, Collegium Medicum, Jagiellonian University, Ul. Grzegorzecka 16, PL-31-531 Cracow, Poland (Dr. M. Kłys).

Aachen: Institute of Forensic Medicine, Klinikum RWTH, Aachen University of Technology, Pauwelsstraße 30, D-52057 Aachen, Germany (Prof. Dr. M. Bogusz).

Altogether, 13 columns of the same type and brand (125 mm × 4 mm I.D. LiChrospher 60 RP-Select-B, 5 μm; from E. Merck, Darmstadt, Germany), but from three different batches where tested in these laboratories. Table 7.1 contains an overview of the columns tested.

Table 7.1: Overview of batches tested in the three laboratories

Laboratory	Batch B_I	B_{II}	B_{III}
Groningen	3	1	1
Cracow	3	-	-
Aachen	3	1	1

7.2.2 Equipment

In **Groningen** and **Cracow**, the low-pressure gradient systems used consisted of: a Merck-Hitachi L-6200 gradient pump; a Merck-Hitachi AS-2000 autosampler; a Merck-Hitachi TS-6200 Column thermostat; and a Merck-Hitachi L-4500 Diode-array detector (all from E. Merck, Darmstadt, Germany). The systems were controlled by, and data were acquired by the Merck-Hitachi D-6500 Diode-array System Manager (DSM) software, running on a 386DX-40 (**Groningen**) or a 386SX-40 (**Cracow**) personal computer, equipped with an AT-GPIB/TNT (IEEE 488.2 compatible) Interface board (National Instruments, Austin, TX,USA). The low-pressure gradient system used in **Aachen** consisted of a Merck-Hitachi L-6200 gradient pump, a Merck-Hitachi AS-2000 autosampler (both E. Merck, Darmstadt, Germany), and a Waters 990 Diode-array detector (Waters, Eschborn, Germany).

7.2.3 Chemicals

Solvent A was a 0.025 M triethylammonium phosphate buffer pH 3.0, prepared by diluting 25.00 ml of 1 M triethylammonium phosphate buffer (Fluka, Buchs, Switzerland) to 1000 ml with HPLC-grade water. Before analysis, solvent A was filtered over a 0.45 μm membrane filter (Schleicher & Schuell, Dassel, Germany). Solvent B was Lichrosolv acetonitrile, gradient grade (E. Merck, Darmstadt, Germany).

The 1-nitroalkanes: nitromethane, nitroethane, 1-nitropropane, 1-nitrobutane, 1-nitropentane, and 1-nitrohexane, were obtained from Fluka (Buchs, Switzerland). 1-nitroheptane, 1-nitrooctane, 1-nitrononane, 1-nitrodecane, 1-nitroundecane, and were obtained by synthesis from the corresponding 1-bromoalkanes according to [144]. A stock mixture was prepared containing 5 μl of each 1-nitroalkane in 1 ml of methanol. Before injection, this mixture was diluted 10 fold with acetonitrile (*i.e.* solvent B).

Calibration mixture A (for acidic and neutral substances) consisted of paracetamol, barbital, brallobarbital, secobarbital, clobazam, indomethacin, prazepam, and phenylbutazone, all 0.1 g·l^{-1} in a mixture of 50% solvent A and 50% solvent B. Calibration mixture B (for basic substances) consisted of morphine sulfate·5H_2O, chloroquine diphosphate, cocaine, diphenhydramine HCl, amitriptyline HCl, thioridazine HCl, meclozine, and amiodarone HCl, all 0.1 g·l^{-1} in a mixture of 50% solvent A and 50% solvent B.

A set of 47 *test substances* was selected. For each test substance, a stock solution containing 2.0 g·l^{-1} was made by weighing about 5-10 mg of the test sub-

stance and dissolving it in an appropriate volume of solvent B, or (if the substance is insoluble in acetonitrile) in an appropriate volume of a mixture of solvent A and solvent B. Appropriate *test mixtures*, each consisted of about four of the test substances, were prepared from the stock solutions and diluted with solvent A to 0.1 $g{\cdot}l^{-1}$.

All calibration standards and reference substances were of pharmacopeical quality, and obtained from different sources. The substances included in the calibration mixtures (*i.e.* the calibration standards) can all be obtained from Sigma (St. Louis, MO, USA), except brallobarbital.

7.2.4 Column system

The column system consisted of the analytical column (see § 7.2.1), equipped with a 4 × 4 mm I.D. LiChrospher 60 RP-Select-B (5 μm) pre-column (E. Merck, Darmstadt, Germany).

7.2.5 Chromatographic conditions

A flow rate of 1.0 $ml{\cdot}min^{-1}$ and an injection volume corresponding to about 2 μg of the reference substance (usually 10 or 20 μl) was used. The temperature was kept constant at 25°C . To avoid bubble problems, a continuous flow of helium gas was lead through the solvents.

The gradient run started with a mobile phase consisting purely of solvent A. From 0 to 30 min. the content of solvent B in the mobile phase was linearly increased from 0 to 70%. Next, the mobile phase composition was kept constant at 70% solvent B and 30% solvent A for 10 min. After this isocratic stage, the composition was returned to the initial values (*i.e.* 100% eluent A and 0% eluent B) linearly in 3 minutes. Prior to the next run, the system was allowed to equilibrate for 10 minutes. As a consequence, a single run took a total of 43 minutes.

7.2.6 Procedures

The RIs of the calibration standards were calculated from their retention times ($t_R(X)$) and the retention times of the bracketing pair of 1-nitroalkanes ($t_R(H_n)$ and $t_R(H_{n+1})$), with $t_R(H_n) < t_R(X) < t_R(H_{n+1})$), using equation 7.1

$$RI_{NO2} = 100\left(n + \frac{t_R(X) - t_R(H_n)}{t_R(H_{n+1}) - t_R(H_n)}\right) \tag{7.1}$$

where n is the number of carbon atoms in the 1-nitroalkane preceding the calibration standard. The RI^cs of the test substances where calculated using equation 6.13.

7.3 Results and discussion

7.3.1 Correction procedure refinement

The RIs of the secondary calibration standards in mixtures A and B were calculated from their retention times and the retention times of the 1-nitroalkanes (*i.e.* the

primary calibration standards), and are given in column RI^b of Table 7.2. From these tables, it can be seen that there were deviations between these values and the values found by Bogusz and Wu [67] on a Superspher 10 RP-18 column, which are represented in column RI^a. Some of the reference substances showed a RI value shift in such a way that these RI values became very close to those of other reference substances. A few were therefore redundant and could thus be left out. Hence pentobarbital, benzoylecgonine and haloperidol, (*i.e.* the substances between brackets in Table 7.2) were omitted. It can also be seen that, compared to Bogusz *et al.* [67,68], more calibration standards have been included, especially in the later part of the chromatogram. This was done to obtain better precision in determining the RIs of substances eluting late.

Table 7.2: RIs of the calibration substances in mixtures A and B on C18 and C8 stationary phases.

Mixture	Standard	RI^a	RI^b	SD^c
A	Paracetamol	243	264	1.6
	Barbital	287	308	1.9
	Brallobarbital	359	371	0.8
	(*Pentobarbital*)	405	(*415*)	(*1.5*)
	Secobarbital	437	443	0.8
	Clobazam	484	488	0.8
	Indomethacin	610	607	0.8
	Prazepam	648	648	4.2
	Phenylbutazone	-	672	-
	Cannabidiol	-	928	-
B	Morphine	198	200	4.5
	Chloroquine	265	282	3.7
	(*Benzoylecgonine*)	395	(*317*)	(*3.1*)
	Diphenhydramine	385	393	0.8
	Cocaine	405	415	1.5
	(*Haloperidol*)	409	(*417*)	(*1.8*)
	Amitriptyline	443	440	2.1
	Thioridazine	504	490	3.2
	Meclozine	601	587	6.4
	Prazepam	-	648	0.8
	Amiodarone	762	680	8.3

a. RIs for C18 stationary phases, from the literature [67]
b. RIs for a C8 stationary phase, determined using 1-nitroalkanes, on 3 different columns and 2 injections on each column in the present study in Groningen
c. Standard deviation (n=6) for RI^b

The RI values of mixture A (given in column RI^b of Table 7.2) were used to calculate the RIs of the acidic and neutral test substances, and the RI values of mixture B (also given in column RI^b column of Table 7.2) to calculate the RIs of the basic test substances. It is important that for the calibration of the RI values using the secondary standards, the proper calibration mixture is being used. Table 7.3 shows that when the RIs of acidic test substances are calculated using calibration mixture B, the values obtained do not deviate much from those obtained using

calibration mixture A. However, the spread is much larger. This is also the case, although to a lesser extent, when for the basic test substances RIs are calculated using calibration mixture A (see Table 7.4). For neutral substances, the use of mixture A or mixture B does not make a significant difference, neither in the value of the RI calculated, nor in the mean standard deviation (see Table 7.5). Because the neutral substances are usually extracted along with the acidic substances, all neutral reference substances are calibrated using calibration mixture A.

Table 7.3: Comparison of RIs for acidic drugs calculated with two different calibration mixtures

	Mixture A		Mixture B	
substance	RI^a	SD^b	RI^a	SD^b
Acetazolamide	271	3.74	271	6.26
Acetyl salicylic acid	353	1.90	353	5.10
Diclofenac	621	3.38	300	17.80
Hydrochlorthiazide	299	4.41	298	8.87
Ibuprofen	622	5.27	625	19.20
Phenobarbital	371	1.24	371	3.03
Salicylamid	328	2.49	328	4.85
Thiopental	486	3.89	487	7.62
Vinylbital	418	3.71	419	9.31
Mean SD		3.34		9.12

Table 7.4: Comparison of RIs for basic drugs calculated with different calibration mixtures

	Mixture A		Mixture B	
substance	RI^b	SD^b	RI^b	SD^b
Alprazolam	459	13.04	460	10.70
Aminophenazone	258	7.24	258	5.91
Caffeine	302	8.99	300	4.09
Clomipramine	466	3.70	467	2.60
Codeine	269	3.49	267	4.41
Diazepam	527	5.22	527	5.20
Flunarizine	584	11.55	580	2.20
Flurazepam	391	4.69	390	1.45
Lidocaine	298	4.50	299	3.82
Lormetazepam	486	1.66	488	3.97
Methadone	446	5.30	446	3.09
Promazine	416	5.50	415	1.48
Tetrazepam	515	8.00	517	5.55
Mean SD		6.38		4.19

7.3.2 Within-laboratory reproducibility, Column-to-column reproducibility

For these parameters, the results obtained in Groningen were considered. The RIs calibrated using the proper calibration mixtures (*i.e.* mixture A for the acidic and neutral test substances and mixture B for the basic test substances) were used.

Table 7.5: Comparison of RIs for neutral drugs calculated with two different calibration mixtures

	Mixture A		Mixture B	
substance	RI^a	SD^b	RI^a	SD^b
Alprazolam	406	4.01	404	2.99
Phenacetin	374	4.75	374	2.00
Phenazone	329	7.94	330	4.30
Phenylbutazone	665	12.76	672	19.30
Codeine	440	7.24	441	4.41
Mean SD		7.34		6.60

Because not all data were available for cyclobarbital, theobromine, tilidine, tolbutamide and warfarin, these substance were omitted from the analysis of variance. Table 7.6 shows the results of the analysis of variance. From these results, it can be

Table 7.6: Analysis of variance for the 5 columns tested in **Groningen**

Source	SS	d.f.	MS	F	p
Column effects	255.9	4	36.98	1.366	0.2452
Substance effects	5123926.9	37	138484.51	2957.175	< 0.0001
Residuals	15827.4	338	46.83		
Total	5140010.2	379			

concluded that there was no significant difference between the five columns.

7.3.3 Inter-laboratory reproducibility

To test the inter-laboratory reproducibility, only the results obtained from the columns of batch A where taken into account. Table 7.7 shows the mean RI values of the test substances and the corresponding standard deviations.

Table 7.7: Mean, Variance and Standard Deviation of the RI data obtained in the three laboratories

Substance	n	Mean	SD	Substance	n	Mean	SD
acebutolol	9	304	2.03	methadone	9	450	3.44
acetazolamide	9	246	5.65	mianserine	9	387	2.33
acetylsalicylic acid	9	337	3.50	normethadone	9	417	5.85
alprazolam	9	462	23.57	oxycodone	9	263	4.36
aminophenazone	9	237	6.04	perphenazine	9	432	4.16
amphetamine	9	234	15.73	phenacetin	9	366	7.58
atenolol	9	233	5.77	phenazone	9	317	13.51
caffeine	9	286	16.13	phenobarbital	9	360	1.22
carbromal	9	396	3.14	phenylbutazone	9	686	48.04
clomipramine	9	476	3.57	promazine	9	407	1.43
codeine	9	252	3.98	propanolol	9	368	1.46
cyclobarbital	7	372	1.92	propyphenazone	9	436	10.77
diamorphine	9	324	1.81	salicylamid	9	310	1.96
diazepam	9	528	9.89	strychnine	9	290	2.61
dibenzepine	9	349	2.42	tetrazepam	9	513	8.48
diclofenac	9	620	3.83	theobromine	7	235	12.19
droperidol	9	373	3.70	thiopental	9	479	6.52
flunarizine	9	595	2.32	tilidine	3	338	1.53
fluphenazine	9	470	3.69	tolbutamide	7	475	1.89
flurazepam	9	385	8.31	trifluoperazine	9	493	3.40
hydrochorthiazide	9	275	5.76	tripromazine	9	496	3.44
ibuprofen	9	620	9.18	vinylbital	9	409	0.90
lidocaine	9	273	11.66	warfarin	3	548	2.08
lormetazepam	9	482	1.36				

In Figure 7.1 the mean RIs are plotted against the accompanying standard deviations. The figure shows a pattern comparable to that of Figure 6.3. Phenylbutazone was regarded an outlier. Including phenylbutazone the mean SD is 6.47. When phenylbutazone is left out the mean SD is 5.57. Figure 7.1 shows that about 75% of the test substances has an SD smaller than 8. In Table 7.8 the results of the analysis of variance are presented. These results show that there is no significant

Table 7.8: Analysis of variance for the 3 test laboratories

Source	SS	d.f.	MS	F	p
Lab effects	565.2	2	282.59	2.678	0.0701
Substance effects	5099029.4	44	115887.03	1098.351	< 0.0001
Residuals	37139.9	352	105.51		
Total	5136975.1	398			

difference between the laboratories.

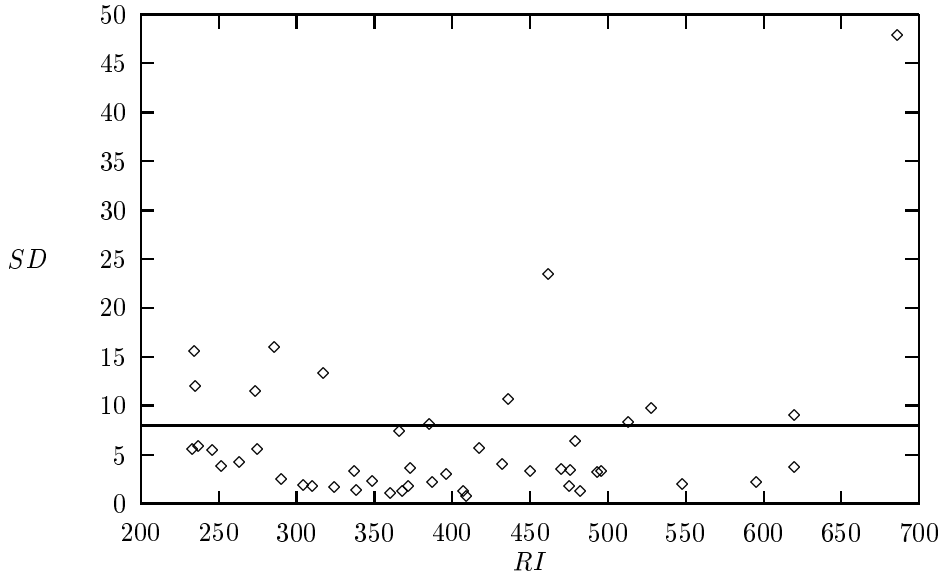

Figure 7.1: Plot of the RI against the accompanying SDs

7.4 Conclusions

The key to the development of recommended methods and useful inter-laboratory databases for STA is the standardization of the analytical methods. Although the instrumental setup has been shown to influence the variability of RIs [145], it obviously cannot be standardized in an inter-laboratory setup. However, the same type of column (*i.e.* a similar stationary phase and the same dimensions) can be used. This was also recommended by Smith *et al.* [146]. For the remaining variability caused by using different columns of a similar type (*i.e.* different batches from the same manufacturer or similar columns from different manufacturers) and different instruments, standardization can be achieved through calibration using the 1-nitroalkane scale and secondary retention calibration standards [71, 143].

A LiChrospher 60 RP-Select-B column is recommend. This column is filled with a C8, base-deactivated packing. According to the manufacturer, this type of column is produced with minimum batch-to-batch variability over a long time compared to other types of columns of the same manufacturer [147]. Thus, due to this guarantee, a database developed using this type of column can be used over a period of years.

After calibration using secondary calibration standards, no significant difference between columns could be demonstrated in a within-laboratory setup. Furthermore, after calibration, no significant difference between RIs determined in three different laboratories could be demonstrated. Hence, the use of secondary calibration standards is a prerequisite for the use of the RP-HPLC RIs on an inter-laboratory

scale. For acidic and neutral substances on one side and basic substances on the other, different calibration mixtures should be used. For most substances (75%), the inter-laboratory SD was less 8 RI-units. Therefore, this value was considered a reasonable estimate of the standard deviation of the RP-HPLC method.